

# The 5' → 3' exoribonuclease XRN1/Pacman and its functions in cellular processes and development

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XRN1 is a 5' → 3' processive exoribonuclease that degrades mRNAs after they have been decapped. It is highly conserved in all eukaryotes, including homologs in *Drosophila melanogaster* (Pacman), *Caenorhabditis elegans* (XRN1), and *Saccharomyces cerevisiae* (Xrn1p). As well as being a key enzyme in RNA turnover, XRN1 is involved in nonsense-mediated mRNA decay and degradation of mRNAs after they have been targeted by small interfering RNAs or microRNAs. The crystal structure of XRN1 can explain its processivity and also the selectivity of the enzyme for 5' monophosphorylated RNA. In eukaryotic cells, XRN1 is often found in particles known as processing bodies (P bodies) together with other proteins involved in the 5' → 3' degradation pathway, such as DCP2 and the helicase DHH1 (Me31B). Although XRN1 shows little specificity to particular 5' monophosphorylated RNAs *in vitro*, mutations in XRN1 *in vivo* have specific phenotypes suggesting that it specifically degrades a subset of RNAs. In *Drosophila*, mutations in the gene encoding the XRN1 homolog *pacman* result in defects in wound healing, epithelial closure and stem cell renewal in testes. We propose a model where specific mRNAs are targeted to XRN1 via specific binding of miRNAs and/or RNA-binding proteins to instability elements within the RNA. These guide the RNA to the 5' core degradation apparatus for controlled degradation. © 2012 John Wiley & Sons, Ltd.

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## INTRODUCTION

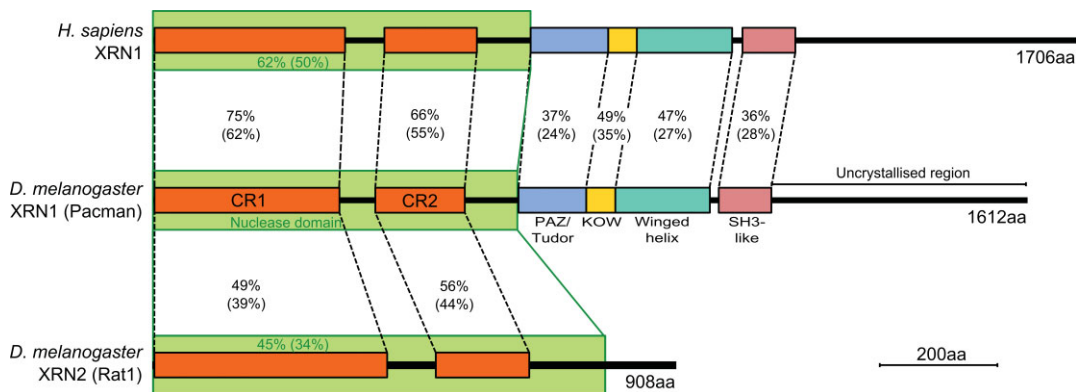
Control of the flow of information from genes to proteins is vital for any organism, and regulation of gene expression has been observed at almost every level from initial transcription, through mRNA processing and translation, to protein degradation. The effect of controlled RNA turnover on gene expression can be extremely significant: e.g., some studies have shown that 40–50% of changes in gene expression occur at the level of RNA stability.<sup>1</sup> In multicellular organisms, it is increasingly evident that degradation of specific mRNAs is critical for

the regulation of many cellular processes, including early development, infection and inflammation, apoptosis, and aging.<sup>2,3</sup> For example, in mice deficient for the RNA-binding protein tristetraprolin (TTP), the stabilities of mRNAs such as tumor necrosis factor- $\alpha$  increase, resulting in a systemic inflammatory syndrome with autoimmunity and bone marrow overgrowth.<sup>4</sup> In contrast, knockdown of the RNA-binding protein HuR (related to the *Drosophila* protein ELAV) destabilizes GATA-3, leading to reduced cytokine secretion.<sup>5</sup> Therefore transcript degradation can be selective and also modulated, suggesting a little studied layer of control of gene expression affecting cellular processes.

The RNA degradation machinery is also intimately linked to other critical cellular and regulatory

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**FIGURE 1** | XRN1 is highly conserved between *Drosophila melanogaster* and *Homo sapiens* and is similar to XRN2 in the nuclease domain (green area), particularly within the two conserved regions (CR1 and CR2, orange areas). Four domains have been identified in the central region of *D. melanogaster* Pacman<sup>12</sup> that are not present in XRN2, but can be found in *H. sapiens* XRN1. Amino acid sequences of the protein domains were compared using Vector NTI Advance 11 (Invitrogen, Carlsbad, California) with the percent similarity shown together with the percent identity (in parenthesis).

processes such as protein translation, nonsense-mediated mRNA decay (NMD), RNA interference, and regulation via microRNAs (miRNAs).<sup>2,3</sup> For example, translational repression followed by transcript degradation is an important control mechanism during specification of the body plan in *Drosophila* and during axon guidance in humans. The molecular mechanisms of nonsense-mediated decay, where aberrant transcripts containing a premature stop codon are degraded, require the recruitment of the core RNA degradation machinery. In addition, knockdown of gene expression by RNA interference or miRNAs utilizes core RNA degradation pathways to eliminate target RNAs.<sup>6,7</sup> Therefore, understanding the molecular mechanisms governing mRNA stability of particular transcripts may also provide insights into other important post-transcriptional processes.

In this review, we aim to outline the mechanisms of mRNA degradation in eukaryotes and then focus on XRN1, the critical exoribonuclease in the 5' → 3' degradation pathway. Recent advances in the understanding of the structural properties and biological roles of XRN1 in a number of organisms will be discussed with particular focus on Pacman, the *Drosophila melanogaster* homolog. We suggest that XRN1 is involved in the specific targeting of particular transcripts involved in development and outline possible mechanisms for this targeting process.

## 5' → 3' EXORIBONUCLEASE CONSERVATION, STRUCTURE, AND MECHANISM OF ACTION

The two main 5' → 3' exoribonucleases found in eukaryotes are XRN1 (Pacman) and XRN2 (Rat1). Rat1 is located in the nucleus and is

involved in ribosomal RNA maturation,<sup>8</sup> transcription termination,<sup>9</sup> and telomere maintenance.<sup>10</sup> XRN1 is predominantly cytoplasmic and is required for the processive degradation of 5' monophosphorylated RNA, such as decapped or cleaved mRNA. Decapped RNA is produced during the normal turnover of mRNA in the cytoplasm, whereas cleaved RNA occurs during RNA interference, degradation via miRNAs and in NMD.<sup>6</sup> XRN1 and 2 show high sequence homology in the N-terminal region, within which the RNase domain is located. There is extensive conservation of the XRN1 N-terminal region across eukaryotes, with greater variation in the C-terminal, which is the segment absent in XRN2 (Figure 1).

The first structural determination of an XRN-type molecule was performed on XRN2/Rat1 from *Schizosaccharomyces pombe*, complexed to its binding partner Rai1. The two well conserved regions in Rat1 form a single domain, of which CR1 forms the active site which CR2 surrounds, facilitating exonuclease activity. Rai1 associates with a poorly conserved region on the C-terminal side of CR2, conferring stability to Rat1 and enhancing its ability to degrade structured RNA.<sup>11</sup>

More recently, the structure of XRN1 has been determined in two organisms, *D. melanogaster* (Pacman)<sup>12</sup> and *Kluyveromyces lactis*.<sup>13</sup> The CR1/CR2 domain containing the active site in XRN1 is similar to that of XRN2, but the large C-terminal of XRN1 is not present in XRN2 (Figure 1). The domains adjacent to CR1/CR2 have been characterized as single PAZ/Tudor, KOW, Winged helix, and SH3-like domains<sup>12</sup>; in *K. lactis* four domains (D1–4) were identified with similar β-barrel structures.<sup>13</sup> The remaining C-terminal region was not crystallized, presumably because it was relatively unstructured.

The nuclease domain, within the N-terminus of XRN1, is able to degrade decapped (5' monophosphorylated) RNA. The 5' phosphate is recognized by a basic pocket formed by four highly conserved residues (Lys93, Gln97, Arg100, and Arg101 in Pacman), from which larger 5' groups are sterically excluded. This explains why XRN1 cannot degrade capped RNA. A helix, known as the tower domain, makes contact with the substrate via His41 and Trp540 and facilitates directional processivity using a ratchet-like mechanism. Therefore the structural characteristics largely explain the specificity of Xrn1/Pacman for 5' monophosphorylated RNA (or single-stranded DNA<sup>14</sup>) together with its processivity. The PAZ/Tudor domain primarily plays a structural role in Xrn1, stabilizing the structure of the catalytic domain, in an analogous manner to the function of Rai1 in stabilizing Rat1. Various deletions or substitutions in the unstructured C-terminus of Pacman or *K. lactis* XRN1 have been shown to impair nuclease activity or affect protein stability, highlighting the requirement of the C-terminal region for XRN1 function, despite its lower conservation compared to the N-terminus. This corresponds with biological evidence where mutations to the C-terminal of XRN1 are detrimental or are unable to rescue the phenotypes of XRN1 null yeast.<sup>14</sup> In *D. melanogaster*, the unstructured C-terminal domain includes a polyglutamine repeat, which may act as a scaffold to organize other proteins around it.<sup>15</sup>

*In vitro*, it has been shown that stable secondary structures, such as extensively base-paired stem loops or G4 tetraplexes can stall the processivity of Xrn1. Although XRN1 has been implicated in G4-tetraplex binding and cleavage,<sup>16</sup> it seems more likely that these observations can be explained by XRN1 degrading the RNA probes up to the G4 tetraplex and then stalling at the stable G4-tetraplex structure. XRN1 appears to be able to degrade structured RNA by pulling the RNA through a channel that is wide enough for only a single strand, which causes duplex unwinding.<sup>12</sup> However, it cannot at present be ruled out that the unstructured C-terminal domain also plays a role in RNA unwinding, either by itself or by recruiting another protein (Box 1).

## DEGRADATION PATHWAYS IN EUKARYOTES

How does XRN1 contribute to mRNA turnover *in vivo*? To answer this question we must understand the eukaryotic mRNA degradation pathways, which are complex and nonlinear. Since actively translating mRNAs are normally held in a circular conformation,

### BOX 1

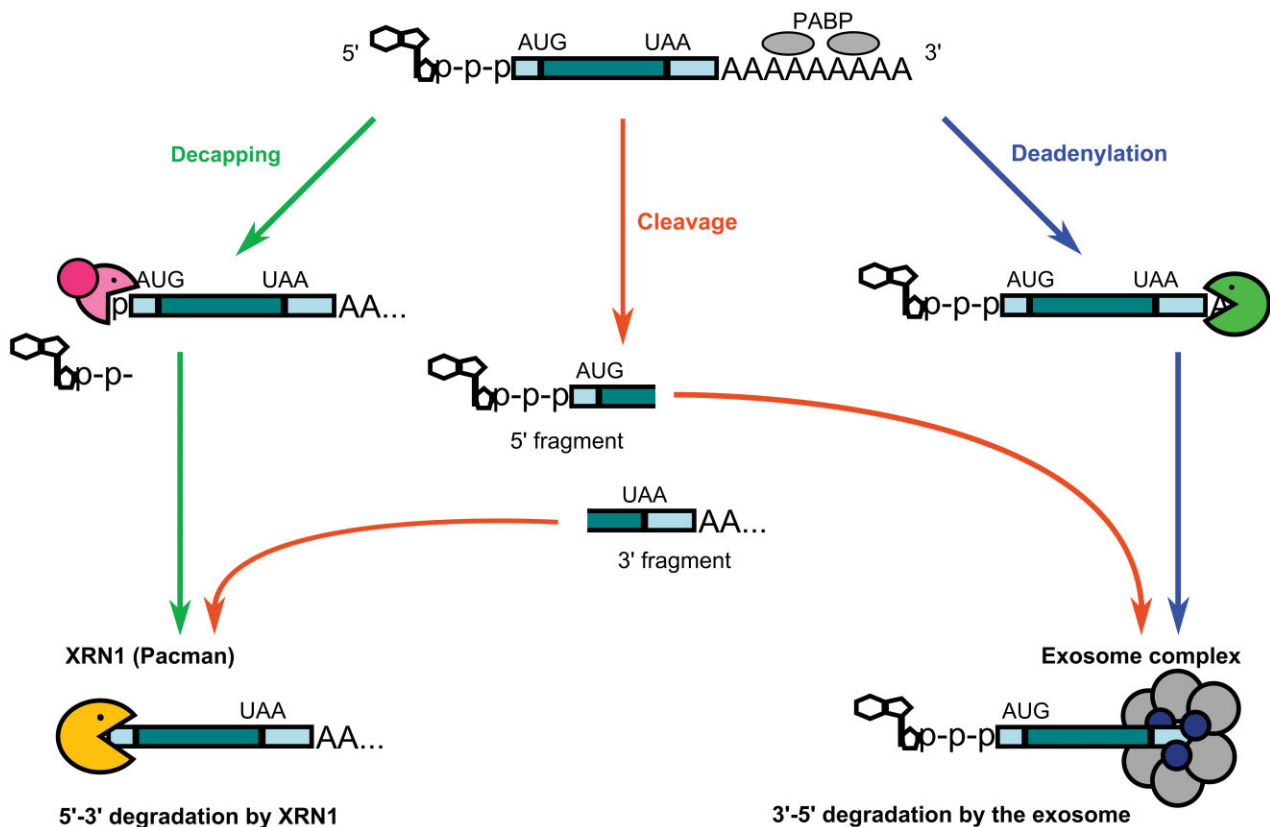
#### DOES A CYTOPLASMIC XRN2 HOMOLOG REPLACE XRN1 IN PLANTS?

No XRN1 homolog is encoded by *Arabidopsis thaliana* or other higher plant species. A number of XRN2 homologs are present, however, two of which are nuclear (AtXRN2/3) and one which is cytoplasmic (AtXRN4).<sup>17</sup> AtXRN2/3 degrade loops excised from miRNA precursors<sup>18</sup> and AtXRN2, like XRN2/Rat1, is involved in rRNA processing.<sup>19</sup> Despite being more closely related to XRN2 than XRN1, the cytoplasmically located AtXRN4 appears to be at least partially a substitute for XRN1. It is not an essential protein, but like XRN1, AtXRN4 degrades the 3' fragment created after miRNA-induced cleavage of mRNAs,<sup>20</sup> and decapped transcripts accumulate when AtXRN4 is mutated.<sup>21</sup> It has recently been shown that the AtXRN4 discriminates between 3' fragments created by miRNA induced cleavage, as only a selection accumulate in mutants.<sup>20</sup> *Atxrn4* mutant plants lack any apparent visible phenotype but were found to be ethylene insensitive as a consequence of the upregulation of EIN3-binding F-box protein1 (EBF1) and EBF2 mRNA levels, encoding related F-box proteins involved in the turnover of EIN3 protein, a crucial transcriptional regulator of the ethylene response pathway.<sup>22</sup>

decircularization of transcripts is the first step in rendering them susceptible to degradation. This can occur in three ways (Figure 2). For normal mRNAs, deadenylation is typically the first step, where the poly-A tail of the transcript is removed to allow access for the exosome, a large complex responsible for 3' → 5' degradation.<sup>2</sup> Alternatively, the decapping enzymes may first decap the RNA. After decapping, which commonly but not exclusively occurs after deadenylation, the transcript is vulnerable to 5' → 3' degradation by XRN1.<sup>2,3</sup> Finally, transcripts can be internally cleaved, producing two products, each of which is vulnerable to degradation by the exosome or by XRN1. Each step of the mRNA degradation pathway is described in detail below.

### Decapping

The best characterized decapping enzyme is Dcp2, which functions together with the decapping activator Dcp1 to remove the 7-methylguanylate cap by hydrolysis.<sup>23</sup> More recently, Nudt16, an alternative decapping enzyme, has been identified in mammalian cells.<sup>24</sup> Both Dcp2 and Nudt16 can show specificity



**FIGURE 2** | Overview of the three pathways used in eukaryotes for mRNA degradation. The circular conformation of mRNA due to the 5' cap interacting with the 3' poly-A tail can be disrupted by removal of the 5' cap (decapping), removal of the 3' poly-A tail (deadenylation), or by endonucleolytic cleavage (e.g., due to RNAi, or nonsense-mediated mRNA decay in some organisms). Decapping exposes the mRNA to degradation by the 5' → 3' exoribonuclease XRN1 (Pacman) and deadenylation allows access for 3' → 5' degradation by the exosome complex. Cleavage creates two fragments, each of which is susceptible to either XRN1 or the exosome.

for particular transcripts or can act redundantly.<sup>25</sup> While Nudt16 is ubiquitously expressed in mammalian cells, Dcp2 is more restricted in its expression, being absent from certain tissues such as heart, liver, and kidney. There are no obvious homologs for Nudt16 in yeast, *Caenorhabditis elegans* or *Drosophila*.<sup>24</sup> After cap removal, the mRNA bearing a 5' monophosphate is vulnerable to degradation by XRN1. *In vivo*, decapping predominately occurs after deadenylation, but has also been found to occur independently of deadenylation for some transcripts, e.g., yeast *EDC1* mRNA.<sup>26</sup>

### ARE-Mediated Decay

AU-rich regions often occur within the 3' UTR of short-lived mRNAs involved in the inflammatory or stress response (e.g., *GM-CSF*, *c-fos*, and *c-myc*). These AU-rich regions have been identified as binding sites for mRNA-binding proteins (like TTP) that can promote transcript degradation by recruitment of the CCR4–NOT complex resulting

in deadenylation of the mRNA and subsequent decapping and degradation of the mRNA by XRN1.<sup>4,27</sup> TTP (as well as BRF proteins) can localize ARE-mRNAs to processing bodies (P bodies; see below), particularly when RNA decay enzymes like XRN1 are limiting.<sup>28</sup>

### miRNA-Mediated Decay

miRNAs are key regulatory molecules which specifically repress protein expression from their target mRNAs. In plant cells, where miRNAs are typically fully complementary to their targets, it is established that binding of miRNAs to their target sequences can result in cleavage of the target transcript by Argonaute proteins and subsequent degradation of the 3' and 5' sections by AtXRN4 and the exosome, respectively. However, miRNA-mediated translational repression or deadenylation followed by decapping may also occur.<sup>29</sup> In animal cells, the mechanisms of miRNA-mediated gene silencing are not clear and may involve a variety of mechanisms, depending on the specific



target or cell type. Recent work shows that degradation of miRNA targets, rather than translational repression alone, is a widespread effect of miRNA-based regulation, and accounts for most of the repression mediated by miRNAs in cell cultures.<sup>29</sup> Although miRNAs can direct endonucleolytic cleavage of fully complementary target transcripts (e.g., HOXB8), this appears to be rare in animal cells, where miRNAs are usually partially complementary to their targets. For these targets, miRNAs usually direct their targets to the 5' → 3' mRNA pathway, where transcripts are first deadenylated by the CAF1–CCR4–NOT deadenylase complex, then decapped by the decapping complex and finally degraded by XRN1. However, it is at present unclear whether decay results from an initial block in translation or through another regulatory pathway, which may be transcript specific or dependent on the cell type (see Refs 29 and 30 for review). Yet another recent model for miRNA-mediated degradation is miRNA cleavage of the target mRNA followed by addition of an oligo U tract at the 3' end of the 5' fragment. 3' U tracts can stimulate 5' decapping, and inhibit 3' → 5' degradation, leading to 5' → 3' degradation of the 5' section of the RNA.<sup>31</sup>

### Small Interfering RNAs-Mediated mRNA Decay

RNA interference requires the binding of fully complementary small interfering RNAs, or longer antisense RNAs (in the case of *C. elegans* and *D. melanogaster*) to the cytoplasmic mRNA target. This binding guides the endonucleolytic cleavage of the target transcript by Ago2 and subsequent degradation of the cleavage products by Xrn1 and the exosome.<sup>30</sup>

### Nonsense-Mediated mRNA Decay

NMD is a RNA quality mechanism which removes aberrant mRNAs that bear Premature Termination Codons (PTCs) or unusually long 3' UTRs. This is important as it prevents the synthesis of truncated proteins which could be detrimental to the cell. The mechanism of NMD differs between yeast, *Drosophila* and metazoans, depending on their complement of Smg proteins.<sup>32</sup> In *D. melanogaster*, which has Smg6 but lacks Smg7, NMD occurs exclusively by Smg6-directed cleavage of the transcript near the PTC followed by degradation of the two cleavage fragments by Xrn1 and the exosome.<sup>33,34</sup> In humans, NMD appears to proceed either by SMG-mediated endocleavage<sup>35</sup> or by exonucleolytic decay from either end (see Ref 32 for review) (Box 2).

## BOX 2

### miRNA DEGRADATION BY XRN1?

Overwhelmingly, research into miRNAs has focused on their biological roles and mechanism of action. Relatively little has addressed the question of how miRNAs themselves are degraded, either at the end of their lives or for regulatory purposes. As miRNAs act cytoplasmically, the 5' → 3' or 3' → 5' RNA degradation pathways are the obvious candidates responsible for degradation of miRNAs. Recent work has suggested that in the case of the unstable human *miR-382*, both the exosome and XRN1 are involved in its turnover, with the exosome playing a more significant role than XRN1, and with no contribution by XRN2.<sup>36</sup> Contrary to this, work in *Caenorhabditis elegans* on a mutant *miR-let-7* has indicated that it is XRN2 that is responsible for its degradation, with XRN1 making no contribution.<sup>37</sup> A further study has shown depletion of either XRN1 or XRN2 can lead to the accumulation of some, but not all miRNA\* 'passenger strands', such as *miR-58\** and *miR-73\**, but not *miR-let-7\**.<sup>38</sup> In addition, 'Small Degrading Nucleases' have been identified as the enzymes that degrade miRNAs in *Arabidopsis*,<sup>39</sup> rather than the cytoplasmic XRN (AtXRN4), which is more closely related to XRN2/Rat1 than to XRN1. The breadth of data concerning this question is currently too limited to draw firm conclusions about the level of involvement of XRN1, or indeed XRN2, the exosome or other nucleases in degradation of miRNAs.

## LOCALIZATION OF XRN1 IN EUKARYOTIC CELLS

XRN1 and other RNA degradation factors vary in cellular location under different conditions and in different cell types, but XRN1 and the exosome do not colocalize in the cytoplasm of cells. XRN1 can be spread diffusely across the cytoplasm, or found localized in P bodies, alongside other proteins that allow for deadenylation, decapping, and 5' → 3' degradation of mRNAs. The localization of XRN1 to discrete cytoplasmic foci was first noted in a mouse fibroblast cell line,<sup>16</sup> and subsequent studies described how other degradation factors such as DCP1/2 and LSM1-7 colocalized in the same particles, leading to the idea that P bodies were the site of mRNA degradation.<sup>3,40</sup> Consistent with this was evidence that showed P bodies appearing or increasing in size in

situations where there is a build up of translationally repressed mRNAs, like during heat shock,<sup>41</sup> when XRN1 function is reduced, such as in *D. melanogaster* or yeast *XRN1* mutants,<sup>42,43</sup> or in human cells with XRN1 depleted.<sup>40</sup> Reducing the amount of repressed mRNA by reducing the rate of transcription results in a reduction in size and number of P bodies, as does inhibiting deadenylation of mRNAs.<sup>43</sup> However, it is possible for mRNAs to leave P bodies and regain translational activity<sup>44</sup> and for degradation to occur without P-body formation.<sup>45,46</sup> This suggests that P bodies, rather than being straightforward RNA recycling centers, act as storage sites for translationally inactive mRNAs, where mRNAs can be degraded or released as required.

Numerous proteins are present alongside XRN1 in P bodies, with the composition varying between organisms and cell types. The deadenylases such as PAN2/3, CAF1, and CCR4, and the associated NOT complex are present in P bodies and first remove the poly-A tail of the mRNA to allow access for the LSM1-7 complex of proteins that bind deadenylated mRNA. The LSM proteins recruit the decapping enzyme DCP2 and its associated factors such as DCP1, PAT1, and EDC3 to remove the 5' m<sup>7</sup>G cap from the mRNA, and the helicase DHH1 (Me31B in *D. melanogaster*) to enhance unwinding of the mRNA to allow XRN1 access (reviewed in Refs 27 and 47). Factors involved in NMD, such as the UPF and SMG proteins<sup>48,49</sup> and transcripts targeted by the RNA-induced silencing complex (RISC) also accumulate in P bodies.<sup>50</sup> EDC3, PAT1, and LSM4 have been identified as P-body scaffolding proteins required for the recruitment and nucleation of mRNA and other factors.<sup>51</sup> XRN1 itself has been found to associate with LSM2/4/8,<sup>52</sup> PAT1,<sup>53</sup> and UPF1/2/3A.<sup>54</sup>

Other RNA granules found within cells contain freshly transcribed mRNAs and co-ordinate localized translation (e.g., germinal granules or neuronal granules), and others process mRNAs released from polysomes (P bodies, as previously described, and stress granules).<sup>55</sup> Stress granules form in response to stress and differ slightly in composition from P bodies, most notably as they contain translation initiation factors and the small ribosomal subunit (see Ref 56 for review). They contain XRN1, but do not contain DCP1/2. Stress granules allow cells to change their translational output in times of stress by aggregating mRNAs not required for the stress response, to allow preferential translation of, e.g., heat shock proteins. Again, however, they are not simple storage sites of mRNAs and initiation factors, as mRNA and protein association can be

transient, and mRNAs can be shuttled between stress granules and P bodies.<sup>41</sup> The role of XRN1 in stress granules is not immediately obvious, as they lack DCP1/2, the action of which is required for XRN1 degradation. However, the RISC is present in stress granules, which may produce a substrate for XRN1 if cleavage/degradation does indeed occur within stress granules.

Neuronal granules are related to P bodies, as they contain DCP1/2 and XRN1, and to stress granules, as translation initiation factors and the small ribosomal subunit are present. They also contain the large ribosomal subunit, NMD/RISC proteins, and translationally repressed mRNAs.<sup>55,57</sup> Neuronal granules are transported to the synapses of dendrites, where they function to change the local translational profile on stimulation. This involves release of the ribosomes, initiation factors and mRNA to form polysomes and may entail degradation and/or translational repression of other mRNAs, as all the machinery required is present (i.e., DCP1/2, XRN1, RISC, etc.).

## FUNCTION OF XRN1 IN VIVO

To understand the biological function of XRN1 *in vivo*, the phenotypes of mutations to XRN1 genes have been studied in a variety of organisms, particularly *Saccharomyces cerevisiae*, *C. elegans*, and *D. melanogaster*. The phenotypes observed in various organisms are summarized later.

### Phenotypes of XRN1 Mutations in Unicellular Organisms

The earliest phenotypic studies on XRN1 were performed in the yeast *S. cerevisiae*. Mutations in *XRN1* resulted in a number of phenotypes suggesting that *XRN1* had a number of different functions. As a consequence, *XRN1* has been referred to by a number of names (Table 1). Disruption of *XRN1* was first shown to decrease growth rate by more than 50%.<sup>58</sup> Around the same time, *XRN1* was identified in a screen for genes that enhance the nuclear fusion defect phenotype of *kar1-1* mutants.<sup>59</sup> *kar1-1* populations form diploids at a lower frequency than wild type and three genes were found in a mutagenesis screen that further reduced the rate of diploid formation; these were referred to as *KEM1-3*, of which *KEM1* was later found to encode the same gene as *XRN1*. The reduced rate of growth was again noted for *kem1* mutant strains, as well as inability to sporulate and hypersensitivity to the microtubule destabilizing compound benomyl (due to increased chromosome loss in the mutant). *XRN1* was further

**TABLE 1** | List of Phenotypes Observed for XRN1 Mutations and Associated Gene Names in Various Organisms

Phenotype/Process Affected	Organism	XRN1 Gene Referred to as	Reference
Reduced growth rate	<i>Saccharomyces cerevisiae</i>	<i>XRN1</i>	58
Nuclear fusion defect and chromosome loss	<i>S. cerevisiae</i>	<i>KEM1</i>	59
Defective sporulation and reduced recombination efficiency	<i>S. cerevisiae</i>	<i>DST2/SEP1</i>	60
Increased chromosome stability during mitosis	<i>S. cerevisiae</i>	<i>RAR5</i>	63
Increased cell size, variation in mRNA, and protein levels	<i>S. cerevisiae</i>	<i>XRN1</i>	61
Microtubule destabilization	<i>S. cerevisiae</i>	<i>SEP1</i>	64
Defects in filamentous growth	<i>S. cerevisiae</i>	<i>KEM1</i>	65
Defects in filamentous growth	<i>Candida albicans</i>	<i>CaKem1</i>	66
Failure of ventral enclosure	<i>Caenorhabditis elegans</i>	<i>xrn1</i>	67
Reduced growth rate	<i>Trypanosoma brucei</i>	<i>XRNA</i>	68
Ethylene insensitivity	<i>Arabidopsis thaliana</i>	<i>AtXRN4</i>	22
Epithelial sheet sealing	<i>Drosophila melanogaster</i>	<i>pacman</i>	69
Reduced male fertility/stem cell maintenance	<i>D. melanogaster</i>	<i>pacman</i>	42
Reduced female fertility/oogenesis	<i>D. melanogaster</i>	<i>pacman</i>	70
Degradation of long, non-coding RNAs (XUTs)	<i>S. cerevisiae</i>	<i>XRN1</i>	62
Increased half life of short-lived mRNAs	<i>T. brucei</i>	<i>XRNA</i>	71

named *DST2* as DNA-strand transfer (spontaneous mitotic recombination) was found to be markedly reduced in mutants, and also referred to as strand exchange protein 1 (*SEP1*) for the same reason.<sup>60</sup> Further work found that *xrn1* mutant cells were larger than wild-type cells, with a reduced protein and rRNA synthesis rates but higher protein levels. The half lives of specific mRNAs were also found to be increased, and it was postulated that the pleiotropic nature of these mutations may be due to the variation in levels of mRNAs and their protein products, rather than *XRN1* physically performing multiple functions itself.<sup>61</sup> More recently, *XRN1* has been implicated in the degradation of long, antisense non-coding RNAs (*XRN1*-sensitive unstable transcripts—XUTs). RNA-seq has been used to identify over 1600 XUTs, the majority of which are antisense to open-reading frames. In strains deficient for *XRN1*, XUTs accumulate, and 273 genes have been identified with reduced transcription due to the increase in antisense XUTs.<sup>62</sup>

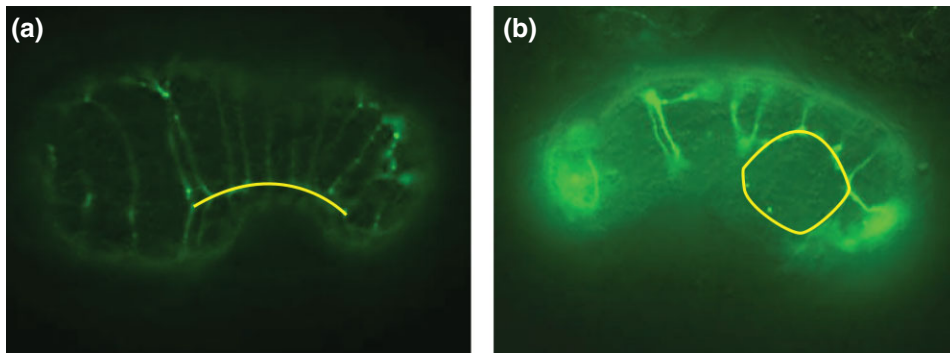
In the kinetoplastid parasite *Trypanosoma brucei*, a transcriptome-wide study has shown that *XRNA*, which is most similar to yeast and human *XRN1*, is involved in the degradation of unstable mRNAs with half lives of less than 30 min. These include messenger RNAs encoding the RNA degradation machinery (exosome, Lsm proteins), nucleotide-binding proteins, and RNA methylases.<sup>71</sup> Previous work showed that downregulation of *XRNA*

and *XRND* are required for trypanosome growth (i.e., multiplication).<sup>68</sup>

### Phenotypes of *XRN1* Mutations in Multicellular Organisms

Development of multicellular organisms requires intricate, defined patterns of protein expression. Careful modulation of the stability of mRNAs that encode developmental proteins is essential to ensure their correct spatial and temporal expression. There are many examples of organisms exploiting mRNA localization, degradation, and translation to control protein expression. As an exoribonuclease, *XRN1* and its homologs are obvious candidates to be involved in rapid degradation of transcripts to prevent their expression. Studies using multicellular organisms also demonstrate that *XRN1* can show specificity for different transcripts resulting in particular phenotypes. *XRN1* phenotypes in metazoans are described below; refer to Box 1 for *XRN1* phenotypes in plants.

This is illustrated by studies in the nematode worm *C. elegans*, where the developmental phenotypes resulting from downregulation of *xrn1* show that correct *XRN1* function is a requirement for post-transcriptional regulation of at least some transcripts at certain stages of development. Our previous work demonstrated that *xrn1* is essential for ventral enclosure,<sup>67</sup> a process analogous to mammalian



**FIGURE 3** | *xrn1* RNAi in *Caenorhabditis elegans* causes a lethal developmental phenotype. In wild-type embryos (a) ventral enclosure completes after hypodermal cells migrate around and fuse on the ventral side. In *xrn1* knockdown embryos (b), migration and sealing fail, which results in a ventral hole.<sup>67</sup>

hind brain closure and wound healing, and dorsal closure and thorax formation in *Drosophila*. Knockdown of *xrn1* by RNAi causes the hypodermal cells that would normally fuse on the ventral side of the *C. elegans* embryo to fail to migrate and fuse together, leaving a hole from which the internal cells protrude<sup>67</sup> (Figure 3).

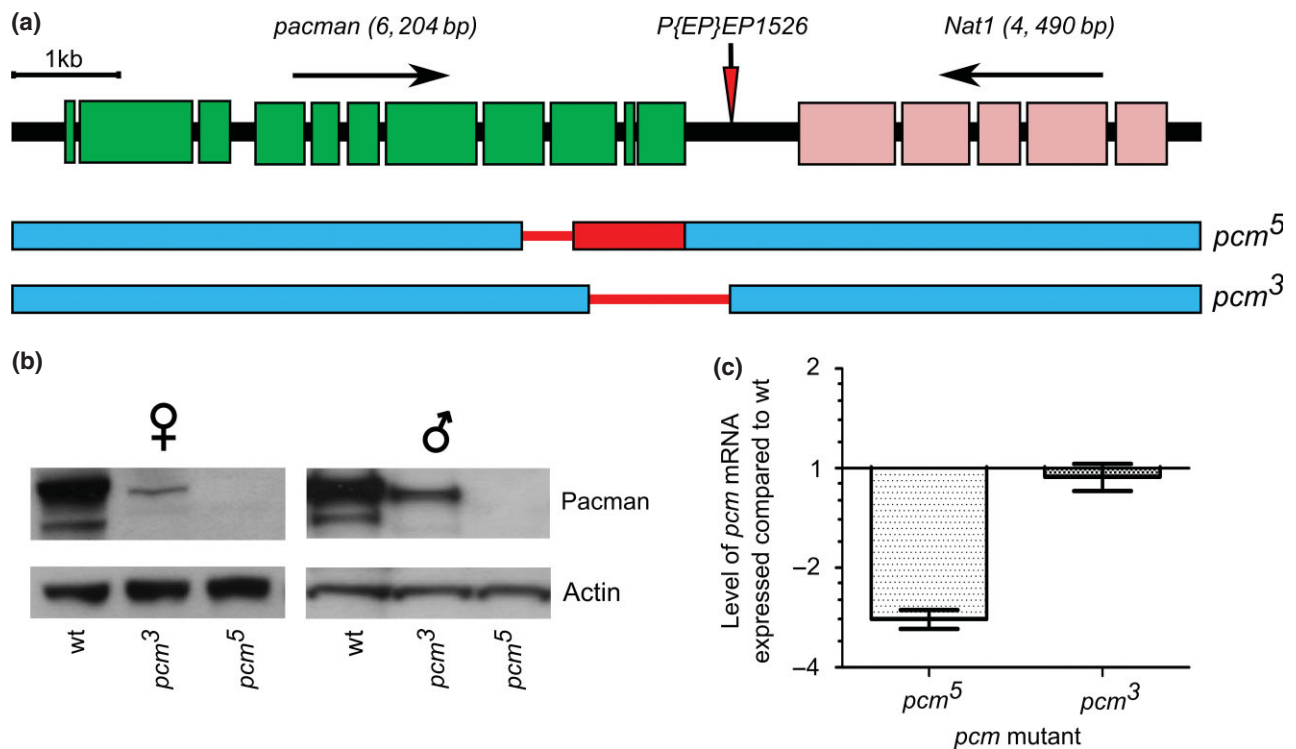
In *D. melanogaster*, previous work from our group using hypomorphic *pcm* mutations (Figure 4, panel A) has revealed specific developmental and adult phenotypes for *pacman* mutants.<sup>69</sup> In these mutants, the level of Pcm protein is reduced significantly compared to wild type (Figure 4, panel B). The level of *pcm* mRNA is reduced in the strongest mutant, *pcm*<sup>5</sup>, by threefold but remains at wild-type level for the weaker mutant *pcm*<sup>3</sup> (Figure 4, panel C). Processes that affect epithelial sheet sealing, which is analogous to ventral enclosure in *C. elegans*, are commonly affected in *pcm* mutants. During development, defects have been observed during dorsal closure in the embryo and thorax closure during metamorphosis. During dorsal closure in wild-type embryos, the two epithelial sheets move over the amnioserosa and meet at the dorsal midline, while in *pcm* mutants the epithelial cells either do not move together, or fail to fuse, and spring back. Thorax closure occurs during pupation as the wing imaginal discs evert and grow toward each other to fuse at the dorsal midline, and also fuse to the leg imaginal discs.<sup>72</sup> In some *pcm* mutants, thorax fusion does not occur completely, and the fly displays a cleft thorax phenotype (Figure 5, panels A and B). A third phenotype related to epithelial cell movement/sealing is that of impaired wound healing in *pcm* mutants, which suffer from reduced survival after wounding compared to wild-type controls.<sup>69</sup> These phenotypes are reminiscent of those seen for JNK pathway mutants in *D. melanogaster*, such as *kayak*,

*hemipterous*, and *puckered*, where dorsal open and cleft thorax phenotypes are common.<sup>72,73</sup> A genetic interaction between *pcm* and *puckered* is evident in double mutants, as a bald patch appears at the anterior of the dorsal midline.<sup>69</sup> The wing imaginal discs appear particularly sensitive to Pcm function, as the effect is not limited to the parts of the discs that form the thorax. The most penetrant phenotype observed in hypomorphic *pcm* alleles presents in the adult wings, which are frequently dull, lacking their normal iridescence, and can be crumpled<sup>69</sup> (Figure 5, panels C and D).

A separate phenotype observed for hemizygotic (male) *pcm* mutants is that of reduced fertility.<sup>42</sup> The strongest hypomorphic allele *pcm*<sup>5</sup> produces half as many offspring as wild-type controls. The testes of *pcm* mutants are the same length as wild type, but are noticeably thinner, by about 25% in *pcm*<sup>5</sup> males (Figure 6, panels A and B). The average number of mature sperm present in the testes of 3-day-old *pcm*<sup>5</sup> males upon dissection was around 3000, almost half the number found in wild-type testes (Figure 6, panel C). The number of sperm and offspring produced by the weaker allele *pcm*<sup>3</sup> were also reduced by roughly a third each. In wild type, Pcm is expressed most strongly in the spermatogonia and stem cells at the tip of the testes, where the initial mitotic divisions occur. Within these cells, Pcm localizes to discrete cytoplasmic foci, determined to be P bodies by colocalization with other P-body proteins, such as Dcp1 and Me31B. Pcm is present at a greatly reduced level in mutants such as *pcm*<sup>5</sup> or *pcm*<sup>3</sup>, and no discrete localization is obvious. However, staining for Dcp1 or Me31B shows an increase in size of the P bodies, by up to 2.7-fold in *pcm*<sup>5</sup>.<sup>42</sup>

Pacman is similarly seen localized to P bodies in female nurse cells, which are involved in oogenesis, and within the egg itself.<sup>70</sup> In *pcm*<sup>5</sup> homozygotes





**FIGURE 4** | (a) Hypomorphic *pacman* alleles were created by imprecise excision of the P-element *P{EP}EP1526* in *D. melanogaster*. Red lines represent deletions (516 bp in *pcm<sup>5</sup>* and 1378 bp in *pcm<sup>3</sup>*). The red box for *pcm<sup>5</sup>* represents a section of the *pcm* gene that is put out of frame by the deletion. (b) The level of Pcm protein is reduced in both whole male and female adults for both *pcm<sup>3</sup>* and *pcm<sup>5</sup>*, with the *pcm<sup>5</sup>* level being almost undetectable by Western blotting. However, genetic evidence shows the *pcm<sup>5</sup>* allele is hypomorphic, so some partially functional protein must be produced.<sup>69</sup> (c) The expression of mRNA from the *pcm* gene in *pcm<sup>5</sup>* and *pcm<sup>3</sup>* was compared to the wild-type level in whole L3 larvae, using a TaqMan quantitative Reverse Transcription Polymerase Chain Reaction (Applied Biosystems, Foster City, California) designed at the interface between exons 3 and 4. The *pcm* mRNA in *pcm<sup>3</sup>* (two biological replicates and six technical replicates) was the same as wild type, while the level in *pcm<sup>5</sup>* was reduced by threefold (three biological replicates and nine technical replicates,  $P < 0.001$ ). Error bars show standard error of the mean.

(females), fewer eggs are produced compared to wild type, and the number of offspring is markedly reduced to just 7% of the wild-type level. In spermatogenesis and oogenesis, the size of P bodies is increased in the *pcm* mutant conditions, an effect often observed when there is a build up of mRNAs that are not being actively translated. In summary, mutations in *Xrn1/pacman* result in specific phenotypes, suggesting that it targets a specific set of mRNAs.

### THE 5' → 3' AND 3' → 5' DEGRADATION PATHWAYS ARE NOT REDUNDANT

Normal mRNA turnover in eukaryotes can proceed from either end of the transcript, suggesting that the 5' → 3' degradation machinery should be able to compensate if the 3' → 5' system is disrupted, or *vice versa*. However, it is clear that the exosome is not capable of compensating for XRN1 in multicellular organisms such as *C. elegans*<sup>67</sup> or *D. melanogaster* (Jones

and Newbury, unpublished data) as knockdown of *xrn1/pcm* results in developmental failures and/or lethality. This shows that each system is individually required for post-transcriptional gene regulation of at least a subset of different transcripts. At the molecular level, it is also clear that there is lack of redundancy as the 3' product of mRNA cleavage (as a result of NMD or RISC activity) can only be degraded by XRN1. When XRN1 is knocked down by RNAi in *D. melanogaster* cell culture, the 3' fragments of reporter mRNAs targeted by RNAi accumulate, while 5' fragments do not. Reciprocally, knockdown of exosome subunits such as Ski2, Rps4, or Csl4, cause accumulation of the 5' fragments, with no effect on the 3' fragments.<sup>6</sup> We have recently observed the same effect in *D. melanogaster* larvae carrying a null *pcm* mutation and *Adh<sup>fm6</sup>*, an allele of *Alcohol dehydrogenase* that undergoes NMD. In the *pcm* and *Adh<sup>fm6</sup>* double mutant, the 3' fragment of *Adh<sup>fm6</sup>* mRNA accumulates (Jones and Newbury, unpublished data). This apparent lack of redundancy



**FIGURE 5** | *Drosophila melanogaster* pacman mutants display a number of developmental phenotypes.<sup>69</sup> (a) A wild-type fly thorax, which forms as the wing imaginal discs grow together and fuse along the dorsal midline during pupation. (b) A *pcm*<sup>5</sup> fly displaying a 'cleft thorax' phenotype where the wing imaginal disc cells have failed to grow/migrate completely across the gap. (c) The wings of a wild-type fly showing their natural iridescence. (d) A *pcm*<sup>5</sup> fly with 'dull' wings that lack iridescence.

between the 5' → 3' and 3' → 5' pathways can most likely be explained by the fact XRN1 and the exosome do not localize together. The 3' fragment of *Adh*<sup>fm6</sup> may never be released from P bodies as it is not a competent mRNA (it has no cap) and therefore will never encounter and be degraded by the exosome, even if it is deadenylated.

## TARGETING mRNAS TO XRN1/PACMAN

*In vitro*, XRN1 is generally indiscriminate in its degradation of 5' monophosphorylated RNAs. However, *in vivo* this is not the case as the resulting phenotypes (detailed earlier) suggest that certain transcripts are more susceptible to XRN1/Pcm degradation than others. It has also been suggested that the controlling step in 5' → 3' degradation, in

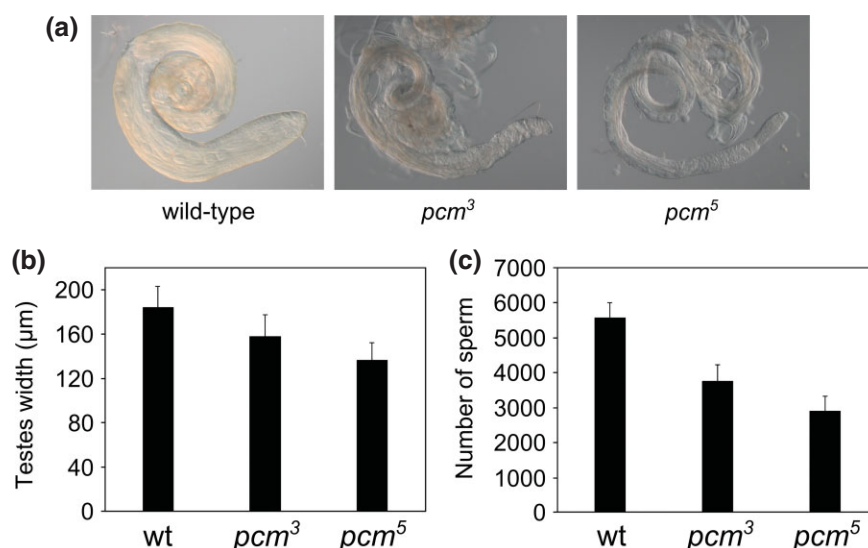
terms of specificity, is the decapping of transcripts. However, the pools of transcripts upregulated in yeast deficient for DCP1 or XRN1 are only 65% similar, showing that degradation pathways controlled by these enzymes are not identical.<sup>74</sup> In addition, the phenotypes of *Dcp1* mutants in *Drosophila* are not the same as the phenotypes of *XRN1/pcm* mutants.<sup>42,69,70</sup> One explanation for this is that there are alternative decapping enzymes in *Drosophila* which have not yet been identified. Another explanation is that mRNAs are specifically targeted to XRN1 and then XRN1 can recruit decapping enzymes (as well as other factors) to degrade the mRNA in the 5' → 3' direction. A model to illustrate this hypothesis is given in Figure 7.

For degradation of particular targets to take place, specific mechanisms are required to target these mRNAs to the core degradation machinery. The available evidence suggests that specific stability/instability elements reside (usually) within the 3' UTR of transcripts. These sequence elements may be recognized at the sequence level and/or may fold into particular secondary structures. A well-known example of an RNA stability element is the AU-rich regions in the 3' UTR of short-lived cytokine and proto-oncogene RNAs (as described earlier). Pyrimidine-rich regions of mRNA 3' UTRs can also promote stability when bound by poly(C)-binding proteins, and are often found in long-lived transcripts.<sup>75</sup> Examples of miRNAs targeting the RISC to mRNAs via specific 3' UTR sequences to cause degradation or translational repression of mRNAs have also been reported. A good example concerns the repression of *dLMO* mRNA by *miR-9a* during *D. melanogaster* wing development (see Ref 7 for review).

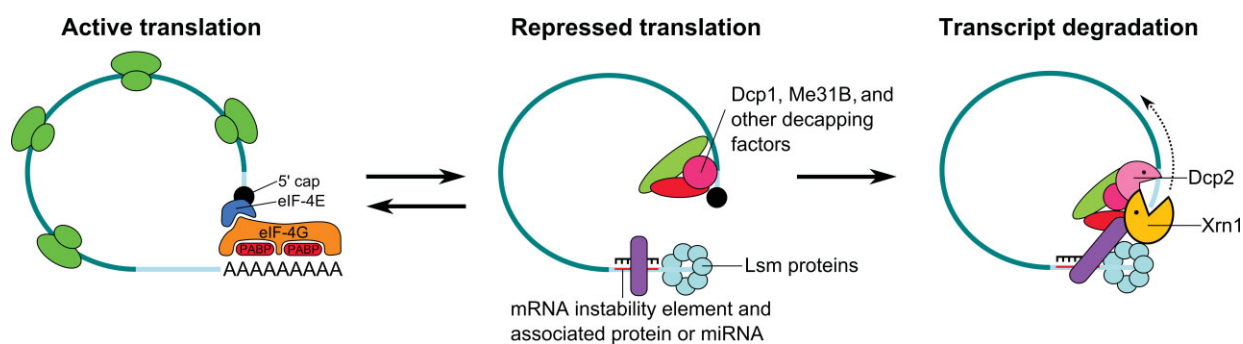
In our model (Figure 7), binding of miRNAs and/or RNA-binding protein(s) to a specific instability element results in assembly of the 5' → 3' degradation complex. Pacman is recruited by this RNA-binding protein and/or miRNA which in turn recruits and activates the catalytic decapping protein Dcp2. The addition of other decapping activators including Me31B and Dcp1 completes the active complex and the target is degraded. Since Pcm has a polyglutamine repeat it is likely to assist in nucleation of P bodies.<sup>15</sup> Presumably, this complex will need to be remodeled to accept another mRNA for degradation. Our laboratory is at present testing this hypothesis using *Drosophila* as a model system.

## CONCLUSION

The 5' → 3' exoribonuclease XRN1 was originally thought to be a passive RNA disposal machine.



**FIGURE 6** | *pacman* mutants display defects in testes morphology and sperm production. (a) The testes of young, hemizygous *pcm*<sup>5</sup> and *pcm*<sup>3</sup> males are significantly disrupted compared to wild type, due to a reduction in width (b). This results fewer sperm being produced (c).<sup>42</sup>



**FIGURE 7** | Proposed model to explain degradation of specific transcripts by XRN1/Pacman. Transcripts targeted for degradation by the 5' → 3' pathway are bound by RNA-binding protein (purple) and/or a miRNA (black) at an RNA instability element (red) within the 3' UTR. This results in assembly of the 5' → 3' degradation complex. XRN1/Pacman (yellow) is recruited by the RNA-binding protein and/or the miRNA and in turn recruits and activates the catalytic decapping protein Dcp2 (pink). The addition of other decapping activators including Me31B and Dcp1 completes the active complex and the target is degraded.

However, our work, and that of others, show that mutations in *XRN1* or its homologs result in specific phenotypes, suggesting that it normally degrades particular subsets of RNAs. In *Drosophila*, the mutant phenotypes suggest that Pacman targets mRNAs involved in stem cell function, cell proliferation, and cell shape change. A key future priority is therefore to identify these target mRNAs, as a first step in the elucidation of the molecular mechanisms involved. Since targeting of particular RNAs is likely to be tissue specific, it will be preferable to carry out these experiments in differentiated cells or to use model organisms. Understanding the molecular processes whereby mRNAs are targeted to the degradation machinery may provide insights into means by which

mRNAs can be artificially targeted for degradation in the cell. Since RNA degradation is intimately linked with translation, these experiments may also shed light on the links between degradation and translation.

Another key consideration is the link between the 5' → 3' degradation pathway and the 3' → 5' degradation pathway(s). Potential links have been little studied in multicellular organisms, yet it is possible that there may be interplay between these pathways. It is also not yet clear how miRNA binding directs the fate of its target mRNA toward degradation or translational repression, or whether RNA-binding proteins are also involved.<sup>7</sup> Understanding the molecular mechanisms underlying this process could

be valuable in novel miRNA therapies. Finally, the signal which triggers mRNAs to stop translation and set out on the degradation pathway is not at all clear.

Although the understanding of RNA degradation pathways has improved greatly in recent years, there is still much to be learned.

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